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Collection, Storage, and Preparation of Human Blood Cells

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Abstract

Human peripheral blood is often studied by flow cytometry in both the research and clinical laboratories. The methods for collection, storage, and preparation of peripheral blood will vary depending on the cell lineage to be examined as well as the type of assay to be performed. This unit presents protocols for collection of blood, separation of leukocytes from whole blood by lysis of erythrocytes, isolating mononuclear cells by density gradient separation, and assorted non-flow sorting methods, such as magnetic bead separations, for enriching specific cell populations, including monocytes, T lymphocytes, B lymphocytes, neutrophils,, , and platelets prior to flow cytometric analysis. A protocol is also offered for cryopreservation of cells since clinical research often involves retrospective flow cytometric analysis of samples stored over a period of months or years.

Keywords

flow cytometry; human blood; leukocyte preparation; mononuclear blood cells; lymphocyte enrichment; cell population isolation; cell population purification

Human peripheral blood samples are common specimens for analysis by flow cytometry. How these specimens are collected, processed, and stored is intimately related to the assay to be performed on each sample. Although the majority of peripheral blood specimens are for leukocyte immunophenotyping, blood is also commonly obtained for the analysis of platelets, neutrophils,, monocytes, rare circulating cells (dendritic cell subsets, endothelial cells, circulating tumor cells, etc), and a variety of microparticles. Many of these assays impose specific requirements for specimen acquisition, transport, and storage. Moreover, although flow cytometry can be used to analyze heterogeneous cell populations, it is often desirable to enrich, purify, or separate cell populations prior to performing flow cytometry, particularly if rare events are to be studied by cytometry. Therefore, it is not possible to present a single, generic protocol for collection, handling, and preparation of all specimens for all assays. As a general principle, cells should be processed as soon as possible after the specimen is obtained and in as close to the native state as possible (i.e., with the least amount of processing possible) in order to reflect the true *in vivo* nature of the cells, and certain general procedures for obtaining blood samples apply.

1. Before taking the sample, read thoroughly the procedure for the specific assay to be performed. Contact the phlebotomist with any information concerning the volume of blood required for the assay and the anticoagulant to be used, as well as any other pertinent information, such as fasting requirements.

2. Procure blood samples from the phlebotomy team as quickly as possible. Maintain blood sample at room temperature unless the specific protocol dictates otherwise. In general, avoid subjecting specimens to extremes of temperature or holding them for prolonged periods of time prior to processing.
3. Be sure to label all specimens properly with type of specimen, time and date of collection, identifier (if appropriate), and test to be performed. Also note the type of anticoagulant used.
4. Store the specimen as appropriate for the assay to be performed. If no specific additives are indicated, maintain blood sample aseptically at room temperature until needed; this temperature is usually acceptable for short-term storage, although this may not be universally true. Gentle rocking may help in preventing cellular aggregation. (Table 5.1.2 provides general recommendations for anticoagulants and storage times for blood samples obtained for a variety of common assays.)
5. Always consider human specimens as potentially infectious and handle using universal precautions including the use of liquid impermeable gloves, lab coats, and protective eyewear.
6. Before collecting the blood specimens, one should get written informed consent from the donor and approval from an appropriate ethics committee.

In some instances, it may be possible to cryopreserve cells for future cytometric analysis. This must be performed with caution, as cryopreservation may affect the expression of some antigens, the loss of specific types of cells and, if not properly conducted, will result in elevated cell death (see Basic Protocol 4).

This unit presents protocols for separating white cells by lysing erythrocytes (see Basic Protocol 2), isolating mononuclear cells using density gradient separation (see Basic Protocol 3), enrichment of lymphocytes by adherence (see Support Protocol 3.1), storage of cells, and a few methods for or enriching specific cell populations. Enrichment of a population of cells prior to cytometric analysis would make analysis of rare cells easier and quicker since fewer irrelevant events would have to run on the cytometer in order to acquire a statistically meaningful number of the rare events. Enrichments of cell populations can be performed by many techniques, as indicated in Table 5.1.1. The most commonly used methods for enrichments are positive and negative magnetic bead-based selections, and example protocols of these will be presented.

BASIC PROTOCOL 1

STORAGE OF WHOLE BLOOD PRIOR TO STAINING

Although it is generally preferable to prepare cells for flow cytometric analysis immediately after collection, in some instances, such as shipping specimens from a distant site, it may be necessary to store blood for brief periods of time prior to staining. Several commercially available reagents can be used to treat blood for storage; however it is imperative that each assay for which the blood is stored is validated on the stored specimens. Essentially one needs to compare assay data from a fresh specimen and the same specimen after storage to

assure similar, if not identical, data are obtained. Two widely used storage reagents are Transfix (Cytomark) and Cyto-Chex (Streck). As an example of using these, a protocol for using Transfix is given here.

Materials

1. Collect peripheral blood into a tube containing an anticoagulant. Remove the top of the blood collection tube and determine the volume of anti-coagulated whole blood within the tube.
2. Pipette an appropriate volume of TransFix into the blood collection tube (the ratio of TransFix to blood should be 1:5). Blood samples should be treated with TransFix as soon as possible after collection, but no more than 6 hours. Blood should not be kept on ice or in the refrigerator before treatment with TransFix.
3. Replace the top on the blood collection tube, ensuring that there is no leakage.
3. Invert the tube at least 10 times (but do not vortex) and store between 2-25°C for as long as 10 days.
4. If refrigerated, allow the stabilised blood sample to return to room temperature (18-25°C) before preparing it for cellular analysis.
5. Examine the sample e.g. using routine flow cytometry evaluation. If determining absolute cell concentrations, the dilution factor arising from the addition of Transfix must be accounted for in the calculations. All antibody conjugates should be validated in association with TransFix prior to use.

BASIC PROTOCOL 2

PREPARATION OF WHITE CELL SUSPENSION BY LYSIS OF ERYTHROCYTES USING AMMONIUM CHLORIDE LYSIS

Although erythrocytes can be separated from mononuclear cells by density-gradient separation (see Basic Protocol 3), many laboratories prefer lysis methods to eliminate erythrocytes from various specimens. Lysis is much quicker than gradient separation and in general leaves the remaining white cell populations relatively unperturbed. Furthermore, the yield of leukocytes from blood by lysis of erythrocytes is much higher than yields from density gradient separations. This procedure, in which erythrocytes are lysed with osmotic shock cell membrane lysis by ammonium chloride, may be used for unstained blood or blood that has already been incubated with monoclonal antibodies. In general, this method will not affect the pattern of staining observed for most lymphoid markers. The viability of white blood cells subjected to this treatment is good.

Materials—Whole blood sample

Homemade RBC lysis buffer as well as commercial RBC lysis buffer without fixative such as ACK lysis buffer (Quality Biological), Optilyse (Beckman Coulter), PharmLyse (BD Bioscience), IOTest3 (Beckman Coulter) Hi-Yield lyse (Life Technologies), Easy-lyse Erythrocyte Lysis Solution (Dako) can be used.

1. Centrifuge the whole blood at 300g for 5 mins and discard the plasma layer, and dilute the remaining cell pack volume with RBC lysis buffer (1:10, volume to volume, or as recommended by manufacturer) and gently rock it for 10 mins at RT.
2. Centrifuge the RBC lysed blood suspension at 300g for 5mins and discard supernatant. Repeat step 2 if RBCs are still visible in the cell pack volume.
3. Resuspend the white cell pellet of leukocytes in PBS and wash the pellet to remove any lysis reagent by centrifugation at 300g for 5mins and discard supernatant.
4. Resuspend WBCs in desired volume of medium or PBS, count them and proceed for further use.

ALTERNATIVE PROTOCOL 2.1

PREPARATION OF WHITE CELL SUSPENSION BY LYSIS OF ERYTHROCYTES USING LYSING REAGENT CONTAINING A FIXATIVE

Commercial reagents such as FACSlyse (BD Bioscience) and RBC Lysis/Fixation Solution (BioLegend), can be used in place of ammonium chloride to lyse erythrocytes. However, as FACSlyse contains a fixative, staining for cell surface markers on leukocytes should be performed prior to lysis of the erythrocytes with this reagent. Stain whole blood with antibodies, then lyse erythrocytes according to manufacturer's instructions. Optilyse (Beckman Coulter) may be used without subsequent washing of the leukocytes.

Materials—Whole blood sample

FACSlyse (Becton Dickinson), ImmunoLyse (Beckman Coulter), or Optilyse (Beckman coulter)

Phosphate-buffered saline

1. Place 200 μ l whole blood sample in a centrifuge tube and add 3 ml fresh 1 $\times$ lysing solution. Incubate 10 min at room temperature.
2. Centrifuge 5 min at 300 $\times$ g, room temperature (22° to 25°C).
3. Discard supernatant. Resuspend cells in 2 ml PBS. Centrifuge 5 min at 300 $\times$ g, room temperature.
4. Discard supernatant and repeat wash with 2 ml PBS.
5. Resuspend cells as necessary for specified assay. Use immediately or store as indicated for assay.

BASIC PROTOCOL 3

ISOLATION OF MONONUCLEAR CELLS BY DENSITY GRADIENT SEPARATION

Density gradient separation methods (Boyum, 1968, 1977) are used when purification of cell populations is required rather than simple removal of erythroid contaminants. Density-gradient separation techniques will not yield as many cells as the simple lysis methods, but they have other distinct advantages. A key advantage of this method for lymphocyte

immunophenotyping is the removal of most granulocytes from the sample. Since these often comprise over 60% of the leukocytes in peripheral blood, removing these cells by density gradient centrifugation decreases the time for acquisition of the sample. An additional advantage is the removal of nonviable cells from the sample. There are modifications of this method to enrich unlabeled cells of interest by using Rosettesep Tetrameric antibody complexes (a cocktail of bi-specific antibodies to preferentially cross link undesired cells to RBCs) (Stem Cell Technologies) which remove unwanted cells by crosslinking and pelleting together with RBCs. This leaves only the unlabeled cells of interest for studies such as cell signaling assays (Phosflow assays), receptor mediated calcium kinetic assays, and so forth.

Materials—1.077 g/ml Ficoll-Hypaque (GE Healthcare) or Histopaque-1077 (Sigma)

Anticoagulated blood in heparin or EDTA

Phosphate-buffered saline

Tissue culture medium

Cocktail of Rosettesep Tetrameric antibody complexes (Stem Cell Technologies), if necessary.

15- and 50-ml conical centrifuge tubes

Centrifuge with swing arm rotor and adaptors for 15 and 50 ml conical tubes.

1. Incubate the recommended volume of Cocktail of Rosettesep Tetrameric antibody complexes (Stem Cell Technologies) with whole blood for 30 mins at RT, if required.

This step is required only, when removal of specific mononuclear subsets are desired to enrich the subset of interest.

2. With a sterile pipet, place the Ficoll-Hypaque solution into a 50-ml conical centrifuge tube, using 2 ml Ficoll-Hypaque per ml blood.

Volume of Ficoll-Hypaque may vary with brand used. Consult manufacturer's recommendations.

3. Mix anticoagulated blood with an equal volume of PBS.
4. Slowly layer the diluted blood over the Ficoll-Hypaque solution by gently pipetting the diluted blood down the side of the tube containing the Ficoll-Hypaque.
5. Centrifuge 30 min at $400 \times g$, 22°C , with no brake.
6. Using a sterile Pasteur pipet, carefully remove the mononuclear cells or desired non-aggregated mononuclear cell subset (if using Rosettesep antibody cocktail), located at the interface between the plasma (upper layer) and the Ficoll-Hypaque (bottom).

7. Transfer the aspirated mononuclear cells to a 15-ml conical tube. Add 10 ml PBS or tissue culture medium and mix thoroughly. Centrifuge 10 min at $400 \times g$, 4°C .
8. Discard the supernatant and repeat wash with PBS or tissue culture medium as needed.

Note: Density gradient separation could be further modified by using 15ml or 50ml Leucosep tubes (Greiner Bio-one) or SepMate tubes (Stem cell Technologies) or similar tubes with incorporated porous barrier tubes. Although the basic protocol and principle of density gradient separation remains the same, there are several advantages offered by Leucosep tubes over the conventional protocol. These tubes provide ease of overlaying diluted blood sample since the porous layer prevents the mixing of blood sample with the separation medium. Centrifugation leads to the separation of unwanted RBCs and granulocytes on the basis of buoyancy underneath the barrier. The enriched mononuclear cells remain above the barrier in the separation medium. This barrier prevents mixing of enriched cells with unwanted cells during harvesting of PBMCs. The combination of Rosettesep and barrier membrane tubes permits purification of unlabelled mononuclear cell subsets without columns or magnets, and with minimum activation of cells.

SUPPORT PROTOCOL 3.1

ENRICHMENT OF LYMPHOCYTES FROM MONONUCLEAR CELL PREPARATIONS BY DEPLETION OF MONOCYTOID CELLS THROUGH ADHERENCE TO PLASTIC

The density-gradient procedure described in Basic Protocol 3 yields a preparation of mononuclear cells that may include monocytoid cells. For most individuals, this protocol provides a specimen with more lymphoid than monocytoid cells. However, in some instances, even further purity of the lymphocytes is desired. This protocol enriches the lymphocyte population by depleting monocytoid cells from the mononuclear cell preparation using adherence to a plastic tissue culture flask.

Additional Materials (also see Basic Protocol 3)—Mononuclear cell preparation (see Basic Protocol 3)

Tissue culture medium containing 10% FBS

75-cm² or 150-cm² plastic tissue culture flask

Humidified 37°C, 5% CO₂ incubator

Additional reagents and equipment for assessing cell viability (UNIT 9.2)

1. Suspend mononuclear cells in tissue culture medium to a final concentration of $1\text{--}2 \times 10^6$ cells/ml.
2. Transfer cell suspension to tissue culture flask.
An appropriate volume for a 150-cm² flask is 50 ml.
3. Incubate cells 1 hr in a humidified 37°C, 5% CO₂ incubator.

4. Gently pour supernatant containing nonadherent lymphoid cells into a 50-ml centrifuge tube. Rinse flask *gently* with 10 ml tissue culture medium and add to centrifuge tube.
5. Check cell suspension manually or with a hematology analyzer to determine percentage of monocytoïd cells remaining in the specimen. If additional depletion of monocytoïd cells is desired, repeat steps 1 to 4.
6. Assess viability of lymphocytes using trypan blue exclusion or flow cytometry (UNIT 9.2).

BASIC PROTOCOL 4

CRYOPRESERVATION OF MONONUCLEAR CELLS

Long term storage of human PBMCs is often accomplished by cryopreservation of the mononuclear cells. This requires isolating the PBMCs using a ficoll gradient (refer to Basic Protocol #3) and then cryopreserving with a freezing medium containing the intracellular cryoprotectant dimethylsulfoxide (DMSO) 10% plus 90% heat inactivated Fetal Bovine Serum (HI-FBS). Cells suspended in the freezing medium are cryopreserved initially in a controlled rate freezer to a temperature of -120°C to minimize cell damage, and are then transferred into a liquid nitrogen tank in the vapor phase of the tank at -156°C ($\pm 20^{\circ}\text{C}$) until thawed for use.

Equipment and Materials Description	Catalog number	Manufacturer
Dimethylsulfoxide DMSO	D128500 or equivalent	Fisher Chemical
Falcon Blue Max 50 ml Polypropylene conical tube	352098 or equivalent	Becton Dickinson Labware or equivalent
Refrigerated Tabletop Centrifuge	75004377 or equivalent	Kendro Laboratory Products or equivalent
HI_FBS	SH30071.03HI	HyClone, GE Healthcare
Various size pipette(s), pipette tips	NA	
Cryogenic vial	5000-0020	Nalgene
Ice bucket	NA	
Cryo-tube rack	NA	
Planer 750Plus Controlled Rate Freezer	Kryo 750 - 30	Planer PLC
Countess cell counter	C10310	Life Technologies
PBS	10010-072	Life Technologies

Procedure

1. Prepare the freezing medium (90% HI-FBS + 10% DMSO). Sterilize the freezing medium using filtration through a 0.22-micron filter. Freezing medium must be prepared fresh at least daily and must be chilled to 4°C prior to use.
2. Label 1.8 ml cryovials and place the labeled cryovials in an ice tray and store in a 4°C refrigerator until needed.

3. Determine the cell count and viability. Do this by mixing 10 μ l of the above cell suspension with 10 μ l of trypan blue, adding to a hemocytometer, and counting on a Countess cell counter. Note: If the original blood volume was greater than 50 ml then the 10 μ l of the above suspension needs to be diluted with PBS in order to reach a cell concentration of approximately 1×10^6 cells in 10 μ l.
4. Turn on the controlled rate freezer and set to wait at 4°C. Move the freezing medium from the refrigerator to an ice bath adjacent to where the cells are being processed.
5. Centrifuge the PBMCs for 10 min at $400 \times g$, 22°C, aspirate the supernatant and resuspend the cell pellet in 1ml of cold freezing medium, mix well by pipetting up and down, then quickly add additional freezing medium to a final concentration of 1×10^7 cells/ml and mix well by pipetting up and down.
6. Aliquoting for cryopreservation: a. For aliquots of 1×10^7 cells: aliquot 1ml samples in freezing medium into 1.8ml labeled cryovials with caps. As cryovials are filled place these in a controlled rate freezer aluminum rack on ice.
7. Transfer cryovials in the aluminum rack to the controlled rate freezer when the chamber temperature reaches 4° C (the green light starts blinking and the controlled rate freezer starts beeping). Start freezing process by pushing the green light button.
8. Freeze according to the following program:
  - (1) 4° C to -6° C with rate -1° C /min
  - (2) -6° C to -45° C with rate -25° C /min
  - (3) -45° C to -20° C with rate +10° C /min
  - (4) -20° C to -45° C with rate -1° C/min
  - (5) -45° C to -120° C with rate -10° C/
  - (6) Transfer sample on dry ice to liquid nitrogen storage

BASIC PROTOCOL 5

ISOLATION OF PERIPHERAL BLOOD MONOCYTES BY GRADIENT CENTRIFUGATION WITH FICOLL-HYPAQUE FOLLOWED BY PERCOLL

Peripheral blood monocytes are isolated from blood by centrifugation through a Ficoll-Hypaque or Histopaque gradient (Denholm and Wolber, 1991) followed by Percoll.

Materials—Venous blood in sodium citrate

Phosphate-buffered saline

1.077 g/ml Ficoll-Hypaque (GE Healthcare) or Histopaque-1077 (Sigma)

10 $\times$ Hanks balanced salt solution

Percoll, specific gravity 1.130 g/ml (Sigma)

Tissue culture medium

50-ml conical centrifuge tubes

10 × 15-cm round bottom polypropylene tubes silanized with Surfasil

1. Mix 30 to 40 ml of blood with an equal volume of PBS. Layer diluted blood over Ficoll-Hypaque solution, using 3 parts diluted blood to 2 parts Ficoll-Hypaque. Centrifuge 30 min at $500 \times g$, 25°C.
2. Using a sterile Pasteur pipet, carefully collect mononuclear cells, located at the interface between the plasma (upper layer) and Ficoll-Hypaque solution (bottom of tube).
3. Transfer cells to a 50-ml centrifuge tube. Add 10 ml PBS. Centrifuge 10 min at $400 \times g$, 4°C.
4. Discard supernatant and resuspend cells in 4 ml PBS.
5. Mix 1.65 ml of 10× Hanks balanced salt solution with 10 ml Percoll. Adjust pH to 7.0 with 0.1 N HCl (~30 µl should be sufficient).
6. Mix 8 ml Percoll solution with 4 ml mononuclear cells in a silanized 10 × 1.5-cm round-bottom polypropylene tube. Mix thoroughly by inverting tube three or four times. Centrifuge 25 min at $370 \times g$, 25°C.
7. Aspirate monocytes by gentle pipetting into a clean test tube. Add PBS or tissue culture medium as needed.

After centrifugation, monocytes appear as a cloudy layer in the top 5 mm of the gradient.

8. Wash the cells in PBS or tissue culture medium.

BASIC PROTOCOL 6

ISOLATION OF B LYMPHOCYTES BY POSITIVE MAGNETIC BEAD-ANTIBODY SEPARATION

Lymphoid subsets can be isolated by either positive or negative selection. Positive selection is more direct, but in some instances the cells of interest may suffer from being manipulated. In those instances, negative selection can be applied by using antibodies that will bind all lymphoid subsets other than the one of interest. The positive selection procedure that follows (based on procedures developed by Rasmussen et al., 1992, and Molday et al., 1977) employs magnetic beads coated with anti-CD19 antibodies to bind B cells. Positive magnetic bead-antibody based separation can be done using whole blood as well as pre-enriched cells depending upon expression of identification CD markers. Using pre-enriched cell populations will help in better yield and purity. Magnetic bead purifications can also be performed using columns and a separation device, or simply in tubes with magnets.

Materials—1% FBS/PBS: phosphate-buffered saline with 1% (v/v) FBS (heat inactivated), 4°C

ACK lysed whole blood or PBMCs

Tissue culture medium with 10% FBS

Detach-a-bead (Life Technologies) or other anti-mouse Fab antibody (optional)

Anti-CD19-coated magnetic beads (Life Technologies/Stem Cell Technologies/Miltenyi)

Magnetic separation device (e.g., Life Technologies/Stem Cell Technologies/Miltenyi)

75-cm² tissue culture flask

15-ml polypropylene test tube

Humidified 37°C, 5% CO₂ incubator

Bind and purify B cells with anti-CD19-coated magnetic beads

1. Thoroughly mix 100 µl of resuspended beads with 10 ml of chilled 1% FBS/PBS in a 15-ml polypropylene test tube. Place tube in magnetic separation device for 5 to 10 min to draw beads to the side of the tube. Gently remove the PBS by pipetting or aspiration.
2. Repeat suspension and separation as in step 1. Resuspend beads in 2 ml of chilled 1% FBS/PBS.
3. Mix 10 ml heparinized blood with 30 ml of chilled 1% FBS/PBS. Place diluted blood in tissue culture flask or polypropylene centrifuge tube. For ACK lysed leukocytes and density gradient separated PBMCs, keep cells in polypropylene tubes in 1ml volume having 5 to 10 million per ml in chilled PBS. Add washed magnetic beads to the flask and incubate 30 min at 4°C with gentle rocking.
4. Separate the bead-cell complexes using a magnet or by placing tube in magnetic separation device for 5 to 10 min.

For this separation, cells may be transferred to a different vessel if necessary.
5. Aspirate and discard fluid from the tube or separation vessel. Remove vessel from magnetic separation device.
6. Resuspend bead-cell complexes in 10 ml of chilled 1% FBS/PBS. Incubate 30 min at 4°C with gentle rocking.
7. Repeat separation and washing with 1% FBS/PBS as many times as desired.

Dissociate B cells from beads by any of several methods, including the following—NOTE: Beads do not necessarily need to be removed from cell prior to downstream applications. For certain applications the antibody, or beads, bound to cell surface antigens can effect subsequent assays and therefore the antibodies used for separation must be removed.

Capping:

- 8a. Suspend bead-cell complexes in 10 ml complete tissue culture medium with 10% FBS in a tissue culture flask and incubate 12 to 24 hr at 37°C in a humidified 37°C, 5% CO₂ incubator.
- 9a. Expose flask to a magnet or place in magnetic separation device for 5 to 10 min.

Exposure anti- mouse Fab antibodies specific for the CD19 antibody, as per Abdallah et al, 2007 (Detach-a-bead, Life Technologies)

- 8b. Suspend bead-cell complexes in 100 to 200 µl complete tissue culture medium with 10% FBS in a polypropylene test tube. Add 10 to 20 µl Detach-a-bead or an appropriate volume of other anti-mouse Fab antibody. Incubate 1 hr at 25°C with gentle rocking.
- 9b. Add 5 to 10 ml tissue culture medium with 10% FBS, mix thoroughly, and expose tube to a magnet or place in magnetic separation device for 5 to 10 min.
- 10. Remove and save suspension, which contains unbound B cells. Repeat capping or exposure to anti-mouse antibodies as desired to remove more beads.
- 11. Centrifuge 10 min at 200 × g, 4°C. Resuspend cells in tissue culture medium for further downstream assays.

ALTERNATIVE PROTOCOL 6.1**ISOLATION OF T CELLS BY NEGATIVE BEAD SELECTION**

For many studies there is a need to work on labeled cells in order to study early cell activation or cell signalling. For downstream assays such as these, investigators need to isolate/purify subsets by depleting undesired mononuclear subsets rather than by specifically purifying a labeled population. For example, isolation of T cells using CD3 will engage the T cell receptor, but enriching T cells can also be accomplished by depleting B cells, NK cells, monocytes, granulocytes, and so forth.

Materials—1% FBS/PBS: phosphate-buffered saline with 1% (v/v) FBS (heat inactivated, 4°C

ACK lysed whole blood or PBMCs see Basic Protocol 1 and Basic Protocol 3.

Tissue culture medium with 10% FBS

Cocktail of Anti-CD19, Anti-CD14, anti-CD56, anti- CD33, anti-CD15, anti-CD16, anti-CD123, and anti-CD235a-coated magnetic beads (Life Technologies/Stem Cell Technologies/Miltenyi)

Magnetic separation device (e.g., Life Technologies/Stem Cell Technologies/Miltenyi)

75-cm² tissue culture flask

15-ml polypropylene test tube

Humidified 37°C, 5% CO₂ incubator

1. Count the cells and add pretitered amount of saturating magnetic beads as per manufacturer's instructions to the single cell suspension and incubate on ice for 30 mins.
2. Place the magnetic column in a magnetic field or adaptor.
3. Load the bead-cell suspension mix on an appropriate size magnetic column (Miltenyi) or place the cell suspension on a magnetic field (Life Technologies/Stem Cell Technologies) and incubate for 5 to 10mins.
4. After the incubation wash the column (approximately 3 to 5ml of 1%FBS/PBS) while on magnetic field and collect the flow through. If not using column, then collect the cells in suspension from the tube, while kept on magnetic field. (Unwanted cells would be bound to the column or will be attached to the wall of tube under magnetic field).
5. Centrifuge the collected flow through or cells in suspension by centrifugation at 350g for 10 mins.
6. Discard the supernatant and resuspend the cell pellet in 1ml of tissue culture medium. Check the purity of collected T cells by flow cytometry.

BASIC PROTOCOL 7

ENRICHMENT OF B OR NK CELLS BY REMOVAL OF T LYMPHOCYTES THROUGH COMPLEMENT-MEDIATED LYSIS

This protocol presents a relatively simple procedure for using negative selection to deplete T cells from mononuclear cell preparations when B cells or NK cells are the population of interest. T cells are lysed with antibody and complement. The subtleties of this procedure revolve around each lot of reagent used. The complement must not be cytotoxic to any of the cells in the absence of specific antibody and the amount of complement and antibody to be used must be determined by titration.

Materials—Rabbit complement (Harlan Laboratories) in tissue culture medium

Mononuclear cell preparation (see Basic Protocol 3)

Anti-T cell antibodies: e.g., anti-CD2, CD3, CD5, or CD7

15-ml conical centrifuge tube

1% FBS/PBS: phosphate-buffered saline with 1% (v/v) FBS (heat inactivated)

1. Place 10⁷ mononuclear cells in a 15-ml conical centrifuge tube. Centrifuge 10 min at 400 × g, 10°C.
2. Remove supernatant and resuspend cells in an appropriate dilution of antibody. Incubate 30 min at 25°C.
3. Centrifuge 10 min at 400 × g, 10°C.

4. Remove the supernatant and resuspend cells in the appropriate concentration of complement in tissue culture medium. Incubate 45 to 60 min at 25°C.

The concentration of complement that gives maximum antibody-specific cytotoxicity is generally 20% to 50% (depending on the source). This can be determined by titration: simultaneously perform the procedure with different dilutions of complement, and then use the concentration that gives minimal nonspecific cytotoxicity and maximum antibody-specific cytotoxicity.

5. Wash the enriched cells from step 4 with 10 ml of 1% FBS/PBS, centrifuging 10 min at $400 \times g$, 4°C.

BASIC PROTOCOL 8

ISOLATION OF NEUTROPHILS BY PERCOLL GRADIENT CENTRIFUGATION

In general, neutrophils require more prompt and delicate handling than lymphoid cells. Specimens more than a few hours old are not optimal for use in neutrophil isolation procedures.

Materials—Blood sample in acid citrate dextrose, formula A

0.6% (w/v) dextran in 0.9% (w/v) NaCl

0.9% NaCl

1.10 g/ml, 1.095 g/ml, and 1.085 g/ml Percoll in phosphate-buffered saline

15-ml polypropylene tubes

1. Mix 45 ml of blood with 10 ml of 6% dextran in 0.9% NaCl. Allow to stand for 1 hr at 25°C.
2. Remove leukocyte-rich supernatant and centrifuge 10 min at $400 \times g$, 4°C.
3. Discard supernatant and resuspend cells in 0.9% NaCl to a final concentration of 5×10^7 cells/ml.
4. Prepare a discontinuous Percoll gradient: transfer 4 ml of 1.10 g/ml Percoll in PBS to a 15-ml polypropylene tube; then overlay this with 3 ml of 1.095 g/ml Percoll and finally with 3 ml of 1.085 g/ml Percoll.
5. Layer 5 ml of cell suspension over the Percoll gradient. Centrifuge 30 min at $700 \times g$, 25°C, in a swinging-bucket rotor with no braking.
6. Sample will contain three bands and a cell pellet. Using sterile polypropylene pipets, collect the two lower bands, which contain the enriched neutrophil fraction. Wash in 0.9% NaCl. Collect the cell pellet and adjust the dilution by PBS for further assays.

BASIC PROTOCOL 9

PREPARATION OF PLATELET-ENRICHED PLASMA

For many platelet assays, the platelets do not need to be purified by density-gradient separation. Platelet-enriched plasma, prepared by enrichment of platelets from peripheral blood (Ault, 1998), is often an acceptable specimen.

Materials—Peripheral blood in EDTA or appropriate anticoagulant

Tyrode's buffer (see recipe)

15-ml conical centrifuge tube

1. Centrifuge 7 ml blood (in collection tube) 10 min at $200 \times g$, 25°C.
2. With a sterile pipet, transfer the plasma layer to a 15-ml conical centrifuge tube. Centrifuge 10 min at $1600 \times g$, 25°C.
3. Remove and discard supernatant. Resuspend pellet containing platelets in Tyrode's buffer or a buffer containing EDTA.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, for suppliers, see **SUPPLIERS**.

Tyrode's buffer

137 mM NaCl

12 mM NaHCO₃

5.5 mM glucose

2 mM KCl

1 mM MgCl₂

0.3 mM Na₂HPO₄

Adjust pH to 7.4 with HCl

COMMENTARY

Background Information

Flow cytometry is a relatively unique technology in that cells need not be purified or separated for the study of a particular subpopulation or clone within a more heterogeneous population of cells. Multiparameter analyses and electronic gating permit flow cytometry studies to be performed without purification of the subpopulations. It is even theoretically possible to analyze peripheral blood leukocytes without prior lysis of erythrocytes using multiparameter methods (Terstappen et al., 1991, Alvarez et al., 2002, Allan et al., 2008).

Indeed, “live” gating techniques where data are gathered during acquisition and prior to analysis may be viewed as “electronic separation” of cell populations, permitting analysis of small populations of cells without their physical separation. Additionally, flow cytometry provides an excellent means of isolating specific cell populations with high purity, if high yield is not an overriding factor.

Although flow cytometry may be used to analyze cells in heterogeneous populations, it is quite often desirable to enrich, purify, or in some way separate populations of cells prior to flow cytometric analysis. The most common example of this is the removal of erythrocytes from peripheral blood prior to analysis of leukocytes. Though not absolutely required, the ease with which erythrocyte removal can be performed, the minimal impact this has on most assays, and the extent of the cytometric problems this avoids makes this removal desirable.

Separation or enrichment of cell populations prior to flow cytometric sorting studies may also be well worth the effort. The throughput and, ultimately, the yield of a desired population of cells may be greatly enhanced by enriching for the target cells in the starting population.

The techniques described in this unit can be used in many different ways. Protocols using antibodies for separation can be applied to a variety of cell types if the appropriate antibodies and cell surface antigens are available. Several of the methods, including the immunosorbent columns and the magnetic bead separation techniques, can be used in either a positive- or a negative-selection mode. Again, the manner in which these techniques are used depends on the cell surface antigens and their corresponding antibodies, as well as any effect these may have on cell function.

Critical Parameters

Numerous points must be considered in using preenriching or separation techniques prior to flow cytometric studies. In addition to yield and purity, possible alterations in cell function must be considered when cells are enriched for subsequent functional studies. Viability and processing time are other issues that may affect the separation or enrichment methods used, or whether any precytometric method should be used at all.

Precytometric enrichment or separation techniques are not without ancillary effects, both beneficial and detrimental. For example, erythrocyte lysis techniques are quite effective in removing mature erythroid cells from blood, but may leave numerous immature, nucleated erythroid cells in the specimen if they were present in the original blood sample. These may be detected in the lymphoid gate by light scatter and may confuse the determination of how lymphoid subsets are distributed (Slade et al., 1988). On the other hand, a separation technique such as Ficoll-Hypaque not only removes granulocytes from the mononuclear cells, it has the added advantage of removing many dead lymphoid cells from the specimen as well; however this is offset by the substantially lower cell yield.

If cell selection methods are used prior to flow cytometric immunophenotyping, care should be taken that these methods do not affect the staining or light-scatter attributes of the cells of interest. For example, use of antibodies in these methods may interfere with subsequent

immunofluorescence staining. Alternatively, granulocytes, for example, may have significantly different scatter properties after cell separation procedures. After any cell separation or preparation technique, particularly if cell surface antigens are to be examined, it is highly recommended that cellular viability be determined. Cells should also be thoroughly washed in isotonic buffer to assure the cells are as free of contaminants as possible and in a native condition. The latter may be of particular concern when staining cells with lectins. Lectins bind carbohydrate structures on the cells, and this may be hindered by residual sugars from the medium or from density gradients.

One last note concerning the handling and preparation of human specimens for flow cytometric analyses: All human-derived material should be considered potentially infectious and should be handled with appropriate caution! The precise precautions taken—e.g., whether specimens are prepared under a laminar flow hood—may vary somewhat from laboratory to laboratory. Certain universal precautions, such as wearing of latex gloves, disinfecting of countertops and equipment with bleach solutions, and the use of face shields should always be followed. No specimen from a human, even a “normal control,” should ever be considered “safe.” Fixation of cells with formalin or paraformaldehyde after staining reduces the infectious potential of human specimens considerably, yet prudent precautions are still advised even when working with fixed specimens.

Troubleshooting

In attempting to troubleshoot the procedures presented in this unit, it is critical that an initial assessment be made of the specimen (cells) to be purified or enriched. The percentage of cells of interest in the initial specimen, the viability of these cells, and the absolute number of cells of interest should be determined prior to any of the procedures. Similarly, the types and numbers of unwanted cells contaminating the initial specimen should be determined. In general, this may best be accomplished by flow cytometric immunophenotyping together with manual or automatic cell counts.

There is usually a trade-off between obtaining maximum purity and maximum yield in isolating cells of interest. This should be borne in mind when separating cells and whether purification (maximum purity) or enrichment (maximum yield) is the ultimate goal. This goal is obviously determined by the final use of the cells and the influence of the remaining, contaminating cells in preparation in the final application.

Unwanted contaminants in the final cell preparation can be the result of any of a number of factors. Assuming protocols are followed precisely and the beginning specimen was typical, a common reason for failure in purification or enrichment of cells is reagent failure. This may be due to substandard lots of reagents, improper storage, or the use of inappropriate or expired reagents. If possible, all reagents should be checked (at least grossly) prior to use. Such checks might include examining the reagent bottles for obvious contamination, reagent expiration date, and any unusual discoloration or precipitate. A second common failure is simply mistakes made in following a protocol, such as inaccurate pipetting or centrifugation speeds.

It is also crucial in most instances to assess the viability of separated cells. This may be easily done by any number of methods (e.g. see *UNIT 5.2*). Additionally, viability should be determined prior to separation procedures. Low initial viability may greatly alter purification, even if viable cells are not needed in the final specimen. Low viability in the final specimen may be due to the presence of cytotoxic agents, such as preservatives, in one of the reagents. A reagent to be considered is the fetal bovine serum, which may not have been properly heat inactivated. Alternatively, poor viability may be due to overly harsh treatment of the cells (e.g., centrifugation).

Anticipated Results

The yield and purity from each of these methods is different. As stated above, before using any of these separation procedures is used, a decision must be made whether this is to be a purification or enrichment process. The resulting yields must be anticipated and evaluated in this light.

Time Considerations

The length of time required to separate cells by these procedures ranges from several minutes to many hours. With most procedures, optimal results will be obtained if cells are processed as soon as possible after collection from the donor. This will minimize problems with viability and any possible alteration of the cell surface antigens.

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TABLE 5.1.1

Cell enrichment methods

Method	Example	Technical Description	Reference
Density gradient	Enrichment of mononuclear leukocytes	Centrifugation of cells through density gradients to separate or enrich cells	Boyum A, 1968; Boyum A, 1977
Erythrocyte lysis	Enrichment of leukocytes	Lysis of RBCs by osmotic shock	Morgensen and Cantrell, Pharm Therap. 1977; 1:369-383
RosetteSep	Enrichment of NK cells from blood	Antibody mediated binding of unwanted cells to RBCs with removal by density gradient separation	Beeton and Chandy; J Vis Exp. 2007;(8):326.
Magnetic bead	Enrichment of mDCs from tumor and blood	Cell enriched (or depleted) by binding to antibody coated magnetic beads	Brocks et al; In vivo 2006; 20(2):239
Complement-mediated lysis	Enrichment of class hybridomas	Unwanted cell removed by complement binding antibody and complement	Faguet and Agee; J Imm Meth 1993;165(2):217
Panning	Enrichment of antigen-specific T cells	Cells enriched (or depleted) by adherence to antibody coated plastic plate	Brousso et al; Immunol Let 1997;59(2):85

CAUTION: When working with human blood, cells, or infectious agents, biosafety practices universal precautions should be followed; see Critical Parameters for further information.

NOTE: All solutions and equipment coming in contact with cells must be sterile, and proper sterile techniques should be used accordingly if cells are to be sorted and subsequently cultured.

Table 5.1.2

Recommended Anticoagulants and Storage Times for Commonly Performed Assays

Assay	Anticoagulant	Time limitation
Lymphocyte immunophenotyping	Sodium heparin or EDTA	Store 72 hr
Myeloid immunophenotyping	EDTA	Use immediately
Neutrophil function	Sodium heparin or EDTA	Use immediately
Platelet activation	EDTA	Use immediately
Platelet markers	EDTA	Use immediately
Reticulocyte enumeration	EDTA	Store 72 hr at 4°C
DNA analysis	Sodium heparin or EDTA	Use immediate for cell-cycle analysis; store 72 hr for ploidy analysis